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High throughput axon injury system for the study of brain injury mechanisms

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The specific aims of this grant were:

1) Construct a high throughput multi-well in vitro axon injury system.
- *a) Develop injury pressure chamber - completed*
- *b) 24 well injury plate - completed*
- *c) Cell free zone fixture for axon injury – created, under testing*
- *d) Uniaxial axon stretch injury – under testing*
- *e) Automatic injury calibration*

2) Characterization of the multi-well injury device.
- *a) Viable culture – completed*
- *b) Control of mechanical stretch*
- *c) Consistency of axonal stretch injury*

3) Validation of multi-well injury system applications.
- *a) High throughput treatment screening and immunocytochemistry*
- *b) Total protein generated from a multi well injury plate*
- *c) High throughput for proteomic analysis*

Summary of accomplishments:

We are pleased to report the successful construction of an operational multi-well injury system. Following is a summary of our accomplishments by specific aim:

Aim 1) Construct a high throughput multi-well in vitro axon injury system.

The high throughput axonal injury system has been constructed and consists of three components: 1) the injury pressure chamber module, 2) the cell free zone fixture for axon injury, 3) the control system hardware, and 4) the control system software, Figure 1.

1) The injury pressure chamber module

This funded project developed a modular axon injury system with two unique applications – a 24 well system for high throughput biochemical analysis and a 6 well system for experiments requiring single sample analysis. Both systems operate under the same principle as outlined in figure 1. Each module is a pressure chamber holds either a 24 well

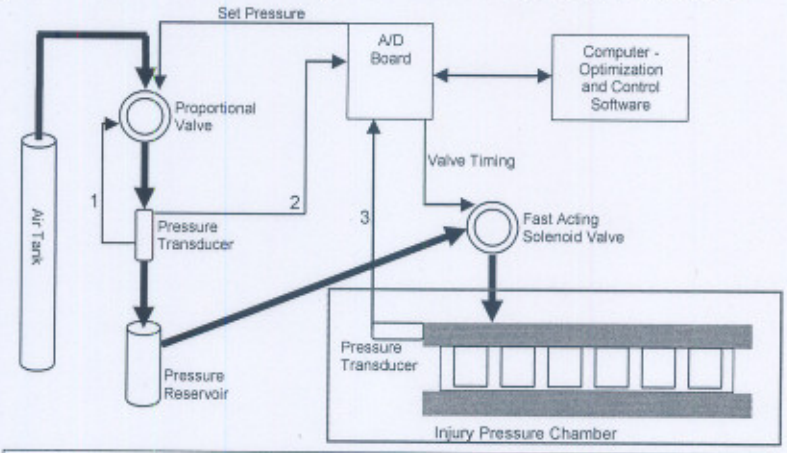


Fig. 1. Schematic of the multi-well high throughput axon injury device. Red outlines the pressure chamber module for a 24 well

The injury device pressure chamber is a sandwich structure consisting of: 1) a top fixture for distributing the pressure pulse, 2) custom made polyetheretherketone (PEEK) cell culture vessels, 3) a silicone membrane sealed to the bottom of the culture vessel and 4) a mask to confine substrate deformation to a defined area, and 5) a bottom fixture to clamp and hold the culture plates; Figure 2. The parts were designed using PRO-E software and manufactured in student design studio by Mr. John Hoinowski.

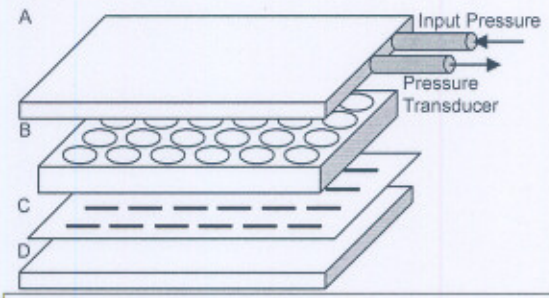


Fig. 2. Schematic of the 24 well pressure chamber. A) A top plate will distribute the pressure-pulse to all 24 wells of the injury plate. An attached pressure transducer will provide feedback. B) The 24 well injury plate. C) Mask restricting deformation of the silicone substrate within the cell free zone. D) Bottom plate fixture that will align and clamp the parts of the pressure chamber above.

1a) 24-well injury plate and pressure chamber module:

A custom made, reusable 24 well culture plate was made from PEEK and designed with the dimensions of a standard 24 well tissue culture plate, Fig 3A. The wells within the plate were made without bottoms so that a deformable silicone membrane could be attached to the base of the plate, on which cortical neurons are cultured. Once the neurons reached the experimental time point, the plate is sandwiched into the pressure chamber (Fig. 3C) and a controlled pressure pulse is delivered to induce stretch injury. The deformation mask is placed below the 24 well plate and restricts deformation of the substrate, Fig. 3B.

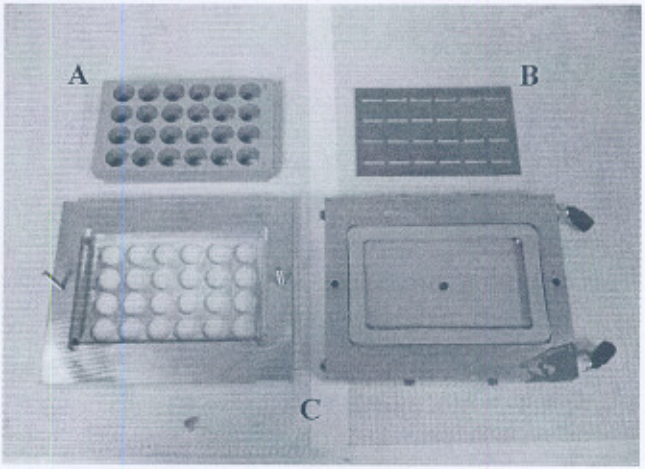


Fig. 3 The 24 well module: (A) Culture plate, (B) Deformation mask and (C) Pressure Chamber

A special technique was developed to attach a deformable silicone membrane to the base of the plate. The silicone must adhere well, not allow for liquid leaks between wells and maintain integrity during rapid pulses of pressure (to stretch the membrane and injury adherent cells).

a) Prior to gluing, the silicone membrane is pre-stretched to remove wrinkles in the membrane and create a small level of tension to provide a secure substrate for cell culture. Figure 4 shows the components of the membrane attachment system. The silicone membrane is sandwiched within a frame and secured with clips. This frame

plate was lightly sanded with 100 grit sandpaper to roughen the surface. The RTV sealant is applied to the base of the PEEK plate with a small roller so that the entire base is evenly coated.

c) The PEEK plate with silicone sealant is placed over the HDPE block and the stoppers along the PVC block maintain its position. A weight is put on the PEEK block to ensure maximum adhesion.

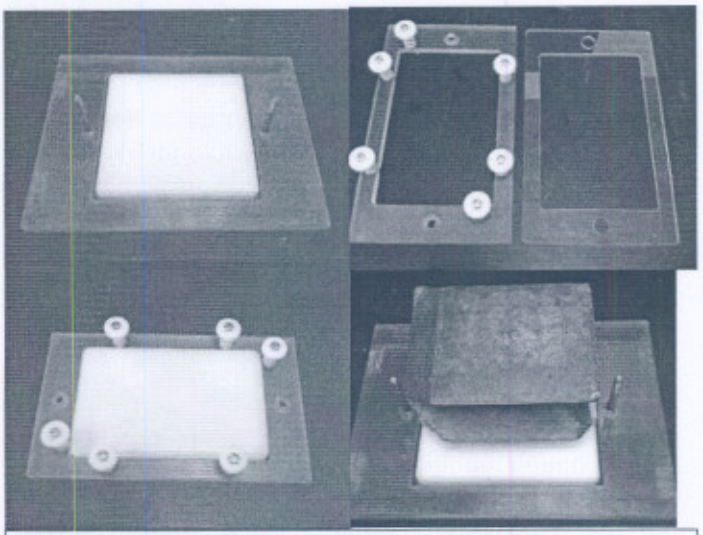


Figure 4, Silicone membrane attachment system

While testing well-to-well consistency (result below), inconsistency was found in the well-to-well deformation. Testing indicated that the problem was due to uneven pre-stretching of the apparatus. The original rectangular geometry of the HDPE block caused irregularity in the pre-stretch of the edge and corner wells. This was remedied by changing the geometry of the HDPE block from a rectangle to a square. In order to gain a quick and simple understanding of the pre-stretch, we replicated (by hand) a sheet of graphing paper onto the silicone membrane. We then took pictures of the membrane before and after stretching to visualize the pre-stretch and found that by changing the geometry of the HDPE block from a rectangle to a square we eliminated the discrepancies in the edges of the membrane.

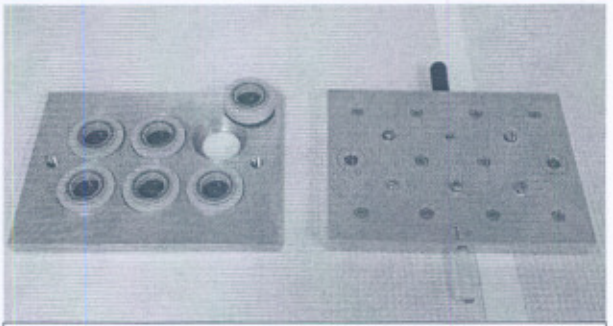


Fig. 5 The 6 well injury module. (A) Pressure chamber base, (B) Single culture vessel with silicone substrate, (C) Chamber top with pressure distribution system.

1b) 6-well injury module and pressure chamber:

The 6 well injury module features individually detachable wells; convenient for single sample studies such as electrophysiological measurements and calcium imaging, Figure 5. As an added feature, this module was designed to minimize internal volume, a major hurtle in the design of the 24 well module. This reduction in volume allows for

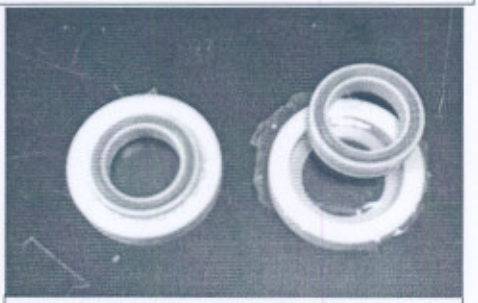


Fig. 6 Exploited view of a single well injury vessel. Snap-fit pre-stretches the membrane evenly.

The silicone membrane is held in place by separable halves of detachable wells; this allows for rapid assembly of the device without the application of the silicone RTV sealant and allows for the device to be cleaned with relative ease, Figure 6. Unlike the 24 well system, these individual wells may be injured separately or any combination up to 6 wells at once. This is useful for time point studies where injured wells could be incubated and analyzed individually or visualized in real time on the microscope.

Fig. 7 Cell plating fixtures

2) Cell free zone fixture for axon injury:

A fixture was developed to easily create a cell free zone with in the culture vessel. Axons later grow into this cell free zone, matching the geometry of the deformation mask for axon stretch injury. We designed and tested several cell plating fixtures, Figure 7. These fixtures are placed into each of the 24 wells - the division through the center prevents cells from sticking to the substrate. Once removed, axons from the developing neurons will transverse the 1.5 - 2mm cell free gap. We have had success with these fixtures in culturing healthy cortical neurons while creating a cell free zone.

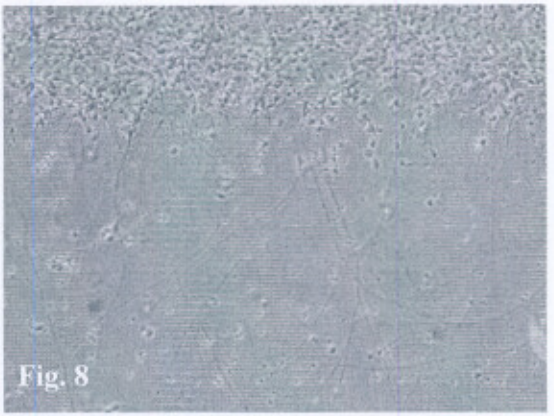


Fig. 8

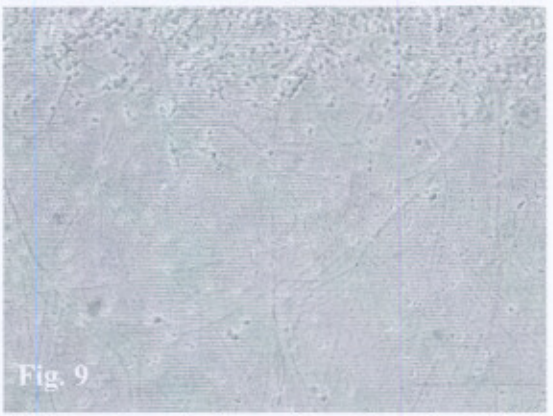


Fig. 9

Figure 8 shows the cell free zone cortical neuron cultures 5DIV (5 days in vitro) and Figure 9 shows the cell free zone of 6DIV neuronal cultures. It can be seen that there are cell bodies in the cell free zone and some residual debris from plating. This may cause problems when measuring total protein generated and performing high throughput proteomic analysis. We are currently experimenting with newly designed plating fixture made of PDMS (Polydimethylsiloxane) rather than ABS (Acrylonitrile butadiene styrene) that we believe will create a "cleaner" cell free zone and is much easier to use. Figure shows our plating fixtures for the 6 well device, 24 well device, and the newly designed PDMS dividers.

The Deformation Mask:

A deformation mask was designed for

deformation of the substrate only cell free zone span across which axons have been grown. This will induce a uniaxial stretch onto adhering axons in culture. For the 6 well device 2 different sized masks (1.5mm and 2mm) have been designed as we are testing the pressure/deformation relationship of this system with a slightly wider gap in anticipation of achieving higher deformation and ultimately more sever injuries.

Testing the limitations of the gap:

Aligning with the concept of a high throughout system, is important to maximize the amount of tissue that we can collect from each 24 well culture plate for analysis. Here, we considered deforming a larger region of the substrate - a larger span of axons. Accordingly, we studied how far we could grow axons across different sized gaps. By placing down small strips of silicone in a cell culture dish we created a cell free zone from 1.5mm to 3mm sizes and images were taken every 2-3 days. We found that axons grew best at a maximum of 2mm, Fig 11.

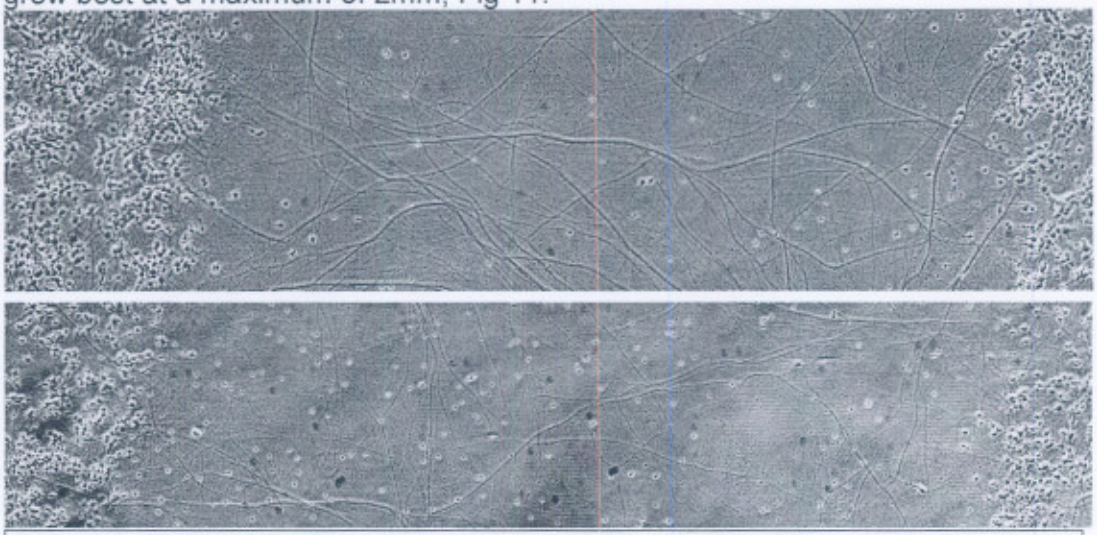


Fig. 11 Cultures across a 1.5mm gap (top) and a 2mm gap (bottom) after 8DIV

3) Control system hardware:

The objective of the control system is to control a reproducible pressure pulse similar to the Penn model - an air pulse of 7 psi within a 20 ms period. This pulse, commonly used for the Penn device, delivers 60% strain at a strain rate of $20s^{-1}$. Figure 12 shows the control system that has been constructed.

The main air tank supplies compressed air, regulated to 150psi to an electronic pressure controller (VSO-EP, Parker Hannifin Corp.). The VSO-EP is a proportional valve that controls the air flow from the main air tank into the reserve air tank. The VSO-EP is user-set to fill the reserve air tank from 0 to 100psi, providing a driving force for the pressure pulse. This valve is controlled by the control input line of the valve which is hooked up to the NI-6009 DAQ card. The internal pressure transducer of the VSO-EP Electronic Pressure Control Unit allows monitoring of the pressure at the reserve tank end via Labview7.1 interface. Safety pop valves of 100psi and 150psi are used to prevent the air tanks from reaching pressures higher than the maximum allowed input pressures into the valves.

Fig. 12

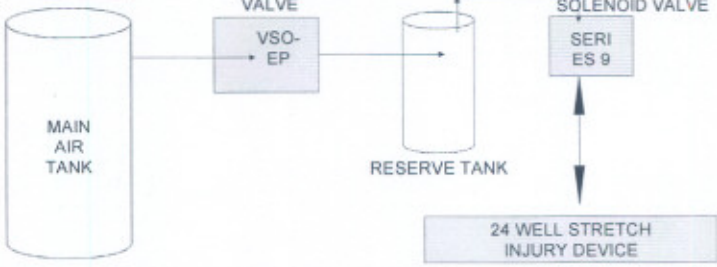
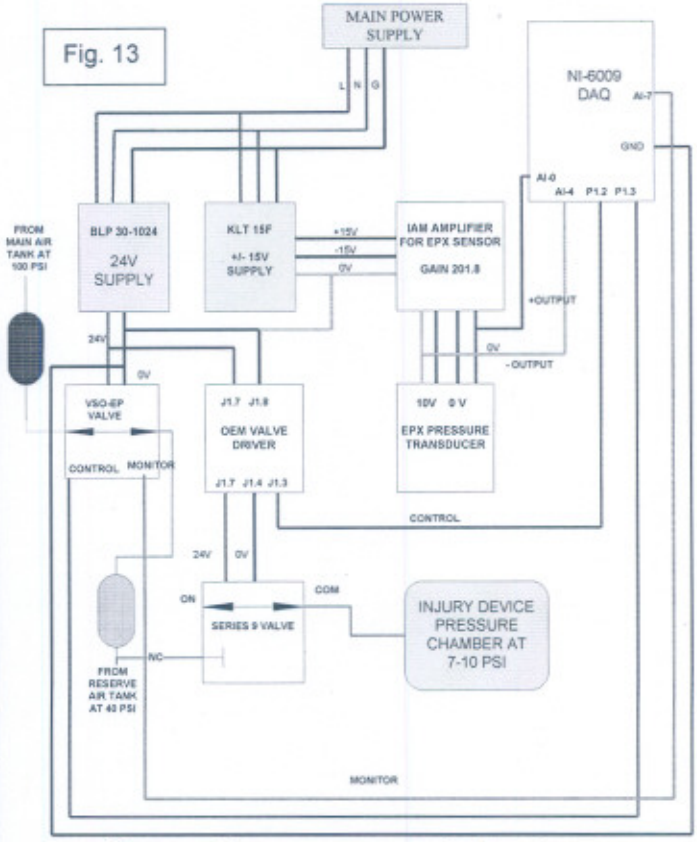
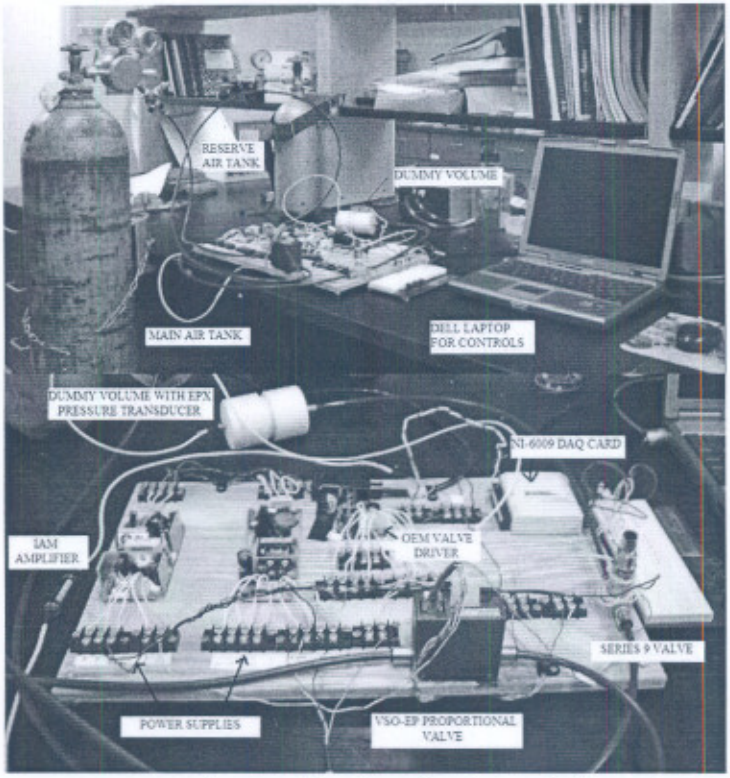
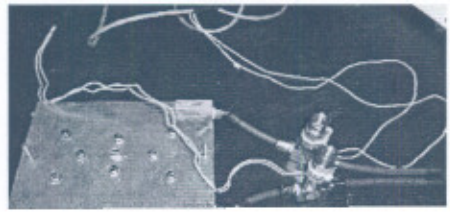


Fig. 13





Once the reserve tank is pressurized to a desired pressure, a control signal is set to drive a three-way normally closed fast acting solenoid valve (Series 9, Parker Hannifin Corp.) to pressurize the injury device pressure chamber. The three-way valve depressurizes the chamber by venting upon closure. The key feature of this valve was the response time, which is less than 5 ms and is the fastest available solenoid valve identified on the market. The Series 9 valve is driven by OEM Valve Driver whose control input line of is hooked up to the NI-6009 DAQ card.



Experimental pressure chamber measurements are recorded using a pressure transducer (EPX pressure transducer, Measurement Specialties). The EPX transducer has a range of 25 psi and the sensitivity is 2.0764 mV/psi. The NI-6009

One issue we found with the 24 well system was achieving a desired pressure pulse that was faster than the University of Pennsylvania device. The reason was the large increase of the pressure chamber volume from translating Penn's single well system to a 24 well system. This massive increase in volume requires a more robust pressure pulse that could not be achieved with just a single valve, so we modified the pressure plate to allow for the use of 2 valves.

4) Control system software:

This system is controlled and monitored through a DAQ card (NI-6009). The software was built on the Labview 7.1 platform. The software has been developed to test the VSO-EP and Series 9 valve operation and the ability of the pressure chamber to reach specified pressures. An *Automatic injury calibration* program has developed to 1) adjust for the opening and closing speed of the valve, 2) the pressure-deformation relationship and 3) the input pressure require meeting the programmed pressure pulse rise times. This routine is executed at the time the unit is turned on. This allows the user to simply input the strain and strain rate desired and the software determines the input pressure and valve timing.

Aim 2) Characterization of the multi-well injury device.

There are three parts to this aim: a) testing culture viability b) testing the control of mechanical stretch and c) testing the consistency of axonal stretch injury.

a) Testing culture viability:

Custom culture environments can affect culture viability. We preliminarily tested the PEEK 24 well plates with attached silicone sheeting with the NG108 neuronal like - cell line, figure 15 top. Cultures were healthy with no sign of toxicity. Meanwhile we established and trained students on cortical cell cultures in our laboratory. We are currently collecting viability data using primary cortical cells from rats, figure 15 bottom. Cells showed excellent viability as quantified below:

Experiment 1

Cell plating density = 200,000 (cells / cm^2).
Average viability (%) = 97.84 (%).

Experiment 2

Cell plating density = 200,000 (cells / cm^2).
Average viability (%) = 97.85 (%).

Experiment 3

Cell plating density = 200,000 (cells / cm^2).
Average viability (%) = 97.20 (%).

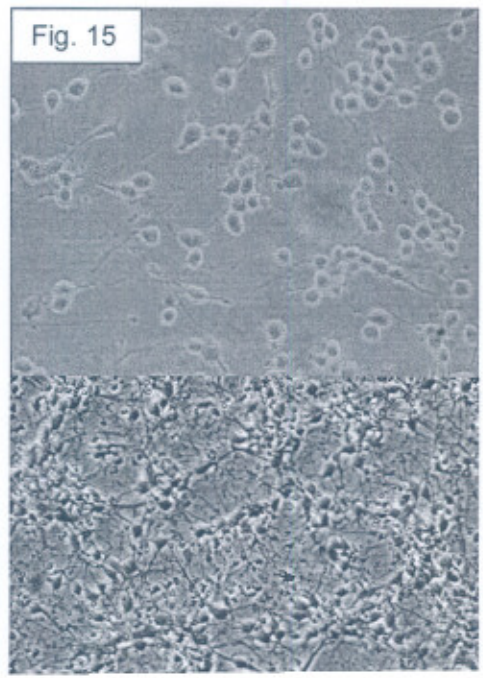


Fig. 15

control system is to produce a reproducible pressure pulse similar to the Penn model - an air pulse of 7 psi within a 20 ms period. Figure 16 shows that our initial objective is being met. Specifically, the increasing slope (pressurization) of each curve is the same and mimicking the Penn device. The trailing end is due to different venting times associated with increasing pressure chamber volumes. Note that the 6 well device can achieve a much faster pressure pulse rise time.

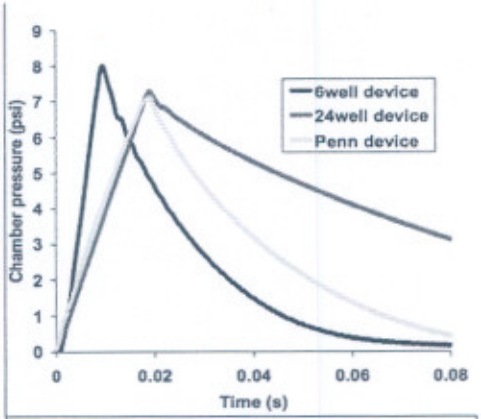


Fig. 16 Pressure pulse curves from each injury module.

Substrate Deformation:

In the assembly of both the 6 well and the 24 well device, the silicone sheeting is pre-stretched equibiaxially to place the sheet under slight tension. This provides a stable substrate for plating cells, removes any directional bias in the supplied sheeting, and ensures the same pretension for each well. Pre-stretch was then analyzed for consistency by measuring the relative displacement of fluorescent beads. Fluorescent beads were adhered to the bottom of the silicone membrane in each well. Images were taken at varying pressures in each well in order to test pre-stretch uniformity and to obtain the pressure deformation relationship of the device. This was done for both the 24 well device (Figure 17) and the 6 well device (Figure 18).

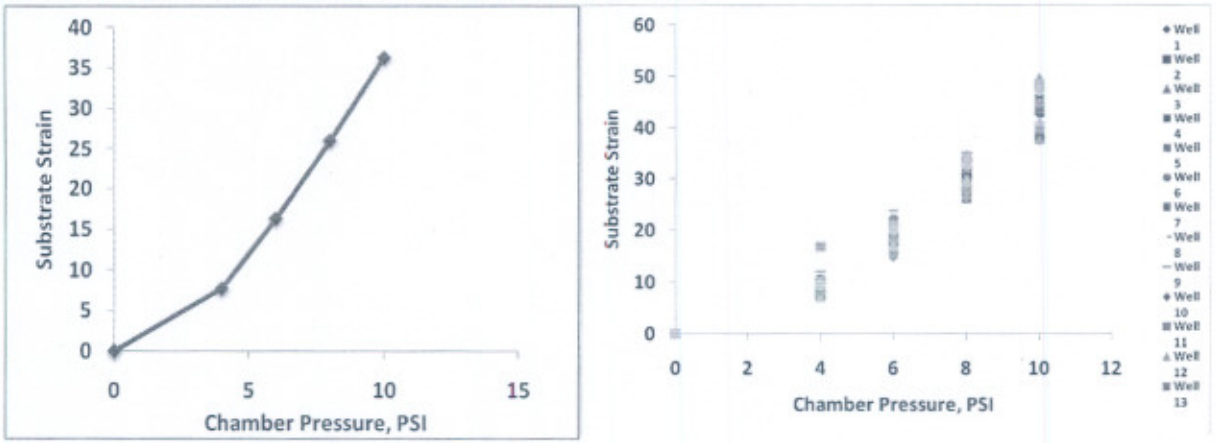


Fig. 17 Pressure – Deformation analysis of the 24 well plate silicone substrates. Left: An averaged curve showing the strain due to the applied chamber pressure. Right: Measurement across all 24 wells showing some variation among wells.

The well images were analyzed using MATLAB by selecting pairs of fluorescent beads that could be identified in all images pertaining to the specified well. Selection of the "bead pair" was based on the ability to visualize "stretch" (displacement between the

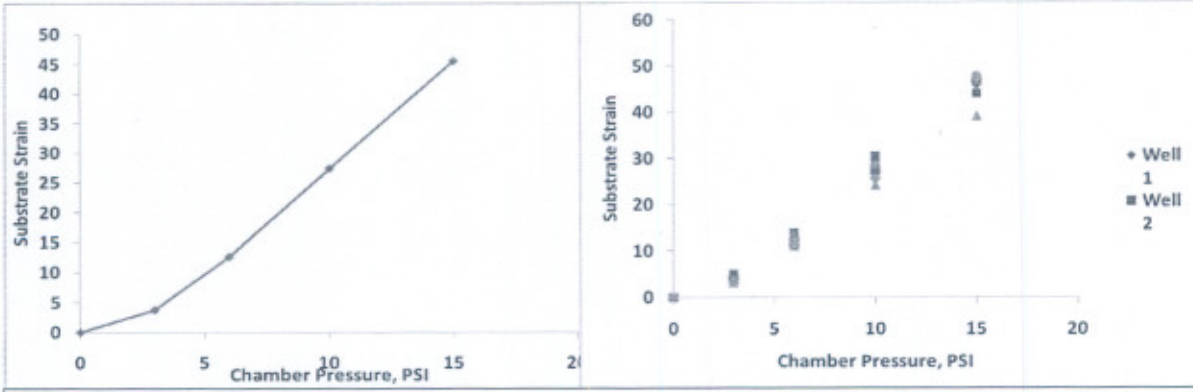


Fig. 18 Pressure – Deformation analysis of the 6 well plate silicone substrates. Left: An averaged curve showing the strain due to the applied chamber pressure. Right: Measurement across all 6 wells showing less variation among wells than the 24 well system.

3) Validation of multi-well injury system applications.

The objectives of this aim were to demonstrate *a) High throughput treatment screening and immunocytochemistry, b) Total protein generated from a multi well injury plate, and c) High throughput for proteomic analysis.* Due to delays getting the 24 well system reliable for experimentation, these goals are currently in progress. In addition, some effort was refocused on the creation of the 6 well device for current experimentation needs.

We have begun by demonstrating objective b) *Total protein generated from a multi well injury plate (see Table below).* The main advantages of a high throughput system is the ability to generate large a mounts of tissue quickly and easily. We ran a total protein assay on 2 plates and generated substantial protein from each plate to proceed with future assays. We are currently in the process of running Western Blot analysis of sodium channel proteolysis (injury induced) using this method. This study aims to determine the mechanism of sodium channel dysfunction (objective c above).

Total Protein Assay	Sample 1 (mg/mL)	Sample 2 (mg/mL)	Sample 3 (mg/mL)	Average (mg/mL)
Plate 1	229.48	220.11	N/a	224.795
Plate 2	285.77	326.09	321.255	311.038